Letter to the Editor

Ct values from SARS-CoV-2 diagnostic PCR assays should not be used as direct estimates of viral load

Dear Editor,

We read with interest the review by Walsh et al.[1] summarizing data on detection patterns and viral loads of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) during the course of infection. We agree with them that determination of SARS-CoV-2 viral load in clinical samples will aid the interpretation of laboratory assays and in the management of isolation and contact tracing protocols, but it should be noted that currently there is no standard measure of viral load in clinical samples. It is becoming common to assume that the C_t values from real-time (quantitative) reverse transcription polymerase chain reaction (qPCR) diagnostic tests are direct measures of viral load, and the use of C_t values has been proposed as a tool to identify those patients who might not be infectious in spite of being positive [2] or to correlate the PCR results with infectivity in cell cultures in order to predict which samples are actually infectious [3,4]. While it is true that C_ts are related to the starting amount of template in the reaction this is not a linear relation and the use of raw C_t values understates the dispersion of the measurements [5]. Another problem is that most diagnostic SARS-CoV-2 qPCR tests are done on suspensions from nasopharyngeal swabs, and these are samples from a surface and have an intrinsic variability that depends on the operator and on the tolerance of the patients [6]. Moreover, the concept of viral load itself is dubious in the absence of a reference mass or volume unit. Finally, the different nucleic acid extraction and amplification systems used by are additional variability sources. For these reasons the assumption that there is a direct relation between the qPCR signal, the amount of virus collected and the amount of virus in the patient's nasopharynx may be misleading and should be taken with care [7].

To illustrate these points, we take advantage of the design a commercial SARS-CoV-2 RT-qPCR that targets two SARS-CoV-2 genes (E and N) in two different reactions (SARS-CoV-2 Real Time PCR kit, Vircell, Granada, Spain). The tube targeting gene N includes an unrelated (and undisclosed) internal amplification control (primers, probe and a target RNA) in the reaction mix, while the tube targeting gene E includes primers and probes for human RNAse P. The housekeeping RNAse P is a ribozyme expressed in many tissues. Specific primers and probes detect both the gene (DNA) and its RNA product and are included for sample quality control in many SARS-CoV-2 and influenza virus commercial assays [8]. To explore the use of human RNAseP to normalize the data we collected the results of a series of 145 randomly selected positive assays from our registers (March and April 2020). In this set the internal control in the N tube had an average C_t of 30 (range 25.3 to 35.5, IQR=2.1 cycles) (Fig. 1A). The human RNAse P control in the

E tube had an average C_t of 28.8 and a broader distribution: range 20.9 to 36.3 and IQR=3.5 cycles (Fig. 1B). The C_t values of the target genes were independent of those of the controls in both cases (Figs. 1A,B, r^2 values not significantly different from zero), meaning that there were no interferences between the target and the control reactions. The variability of the internal controls in the N tubes must be due to experimental errors during the setting up of the PCR reactions, while the higher variability of the human RNAse P controls in the E tubes reflects, in addition, the variations in the amount of material collected with the swabs and in the nucleic acid extraction process.

To correct for sampling variability we used the human RNAse P as a reference to normalize the viral load by the comparative C_t method (ΔCt) [9] that transforms the C_ts into relative loads (ratios of viral target to human target). Fig. 1C shows a plot of SARS-CoV-2 gene E C_ts normalized with the human RNAse P C_t values against the gene E C_ts. The plot shows an inverse linear correlation, which is expected because Ct values reflect, indeed, viral loads, but the dispersion of the data may reach up to four log units (ten thousand-fold) for any given Ct (black arrow). This is not a problem of this particular brand or PCR design, it could be observed in other commercial (TaqMan 2019-nCoV Assay Kit v1, Thermo Fisher Scientific, Waltham, MA, USA) and in-house[10] assays.

Normalization is not as straightforward as suggested by this example. A full characterization of the linear ranges and a calibration using standards[11] should be done for every different target and primer/probe design. Other reference genes might be explored as well, although human RNAseP has been widely used and might enable to exploit the huge amount of data already collected in many laboratories around the world.

Using C_t values obtained in diagnostic PCR reactions as direct measures of SARS-CoV-2 viral loads is simple, but at the cost of introducing errors that cannot be neglected. Normalization using some marker of the cell mass or the mucosal surface sampled should be integrated into commercial diagnostic kits to make the different assays comparable and to evaluate the potential of quantitative PCR for the clinical management of COVID-19 patients.

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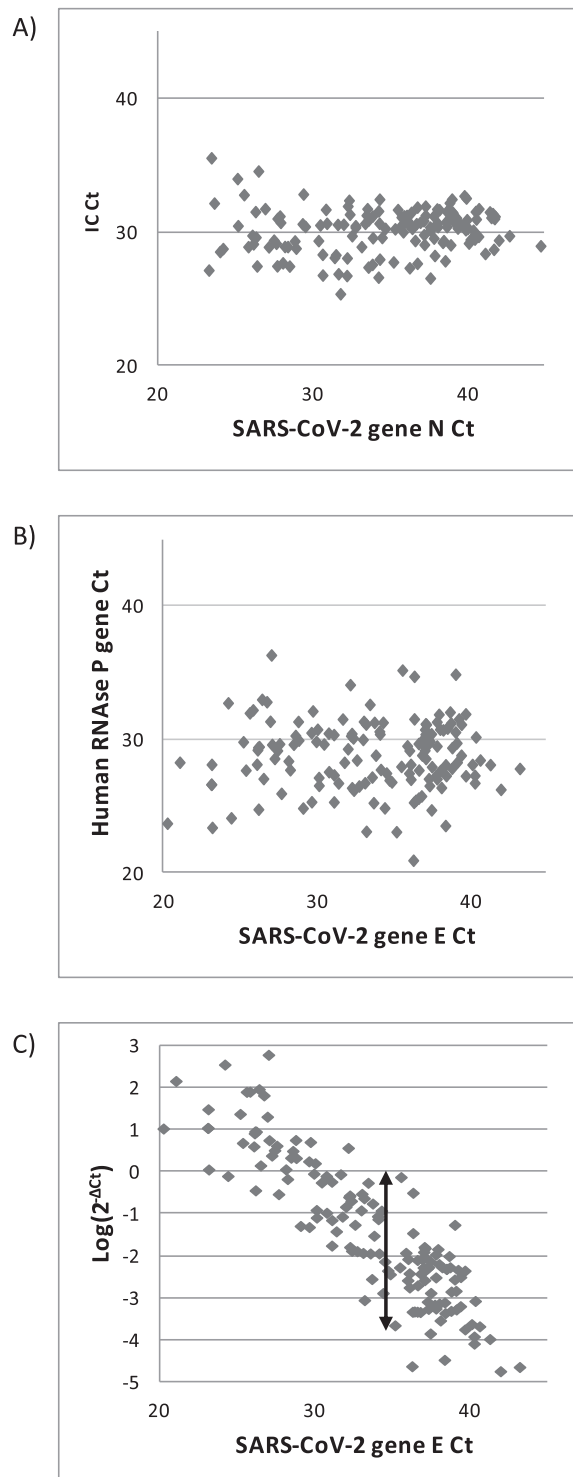
Fig. 1. Analysis of the SARS-CoV-2 Ct values obtained using a commercial RT-qPCR assay (Vircell) in a set of clinical samples. A) C_ts of the Internal Control RNA plotted against the SARS-CoV-2 N gene C_ts ($r^2 = 0.004$). B). C_ts of the human RNAse P plotted against the SARS-CoV-2 E gene C_ts ($r^2 = 0.007$). C) Normalized SARS-CoV-2 gene E C_t values ($\log(2^{-\Delta Ct}) = \log(2^{-(Ct target - Ct reference)})$ plotted against the SARS-CoV-2 E gene. The normalized C_t values are relative loads (ratios of viral target to human target) and are transformed to logarithmic scale for graphical representation. The black arrow illustrates the broad range spanned by any particular C_t value.

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